81. The Tat protein, fragments thereof and/or mutants, as defined according to claim 79, for preventing or treating AIDS, tumors and syndromes associated with HIV infection.--

IN THE SPECIFICATION

Please substitute Fig. 1a enclosed for Fig. 1a as filed.

Please substitute the enclosed specification for the as-filed specification.

Please delete SEQ ID NO. 3 at page 36, lines 14-17 and substitute new SEQ. ID. NO. 3 which appears at page 37, lines 11-17 of the substitute specification.

REMARKS

Reconsideration is respectfully requested in view of the foregoing amendments and the remarks which follow. Applicant by this Amendment has sought to address each of the issues raised by the Examiner and, by so doing, to advance the prosecution of the application to allowance.

With regard to the statement by the Examiner that the claims attached to the substitute specification are different from some of the current claims, it would be most helpful if he would point out those inconsistencies. Based on applicant's examination of the claims, those differences are not readily evident.

A substitute specification is enclosed to correct a variety of spelling errors.

A substitute Figure 1a, panels a-e, is enclosed herewith. Accordingly, the objection thereto has been overcome and should be withdrawn.

An error was discovered in original SEQ. ID. NO. 3. A "T" should have been inserted between positions 62 and 63.

The objection to the specification because the word "control" was misspelled in Table 3 on page 38, has been overcome by the substitute specification where the corrected spelling of the word now appears.

Prior to addressing the rejections raised by the Examiner, it is thought that it would be beneficial to point out the essence or gist of the present invention.

The uniqueness of the present invention resides in the use of biologically active Tat prepared in accordance with the present invention for vaccination against AIDS and related diseases.

"Biologically active Tat" is intended to mean a substantially monomeric Tat protein, obtained by conventional techniques of recombinant DNA, mutants or fragments thereof, that are capable of 1) entering activated endothelial cells or dendritic cells at concentrations up to 10 nM and 2) performing at least one of the following actions: i) activating the proliferation, migration and invasion of Kaposi's sarcoma (KS) cells or cytokine-activated endothelial cells; ii) activating virus replication when added to infected cells as measured by a) the rescue of Tat-defective proviruses in HLM-1 cells after the addition of exogenous protein and/or b) the transactivation of HIV-1 gene expression in cells transfected with a HIV-1 promoter-reporter plasmid; iii) inducing in mice the development of KS-like lesions in the presence of angiogenic factors or inflammatory cytokines.

Only when the above conditions are present is Tat considered to be "biologically active" and that protein is the only one which can be utilized as a vaccine against HIV/AIDS.

As extensively discussed in the prior art section of the specification, Tat is a well-known protein, as are its mutants and fragments (page 2, line 14 bridging page 3, line 16) (see, among the cited references, Yang et al, J. Virol. 70: 4576, 1996; Kashanchi et al., J. Virol. 70: 5503, 1996; Chang et al., AIDS 11: 1421, 1997).

By "native form" of Tat it is intended to mean a Tat protein obtained by conventional techniques of recombinant DNA purified under non-denaturating conditions. In its native form, however, Tat is very sensitive to oxidation and aggregation. Therefore, in conventional preparations, in which no precautions are taken, such as protection from air, light, use of degassed buffer with reducing agents and presence of a carrier protein (i.e. bovine or human serum albumin) (see Ref. 26 of the present application), Tat loses its biological activity as a result of oxidation. Oxidation creates intra- and inter-molecular bonds due to seven (7) cysteine residues present in the Tat sequence (see page 24, lines 21-25 of application). The state of knowledge in the art at the time the instant application was filed was that a native Tat was considered to be in an active form. These so-called "traditional" active forms have been repeatedly studied and tested for activity as immunogens against HIV virus. However the results have been scarce, nonpredictive and often inconsistent. In fact, even though they have demonstrated some immune response *in vivo*, they have been completely inactive *in vitro*. Applicant has been able to prove and establish that oxidized/inactive Tat does not fulfil the definition of biologically active Tat, as reported above.

By contrast, biologically active Tat, as contemplated by the instant claims, is a native protein that is neither oxidized nor aggregated. Biologically active Tat, as defined by

Applicant, is produced and purified by techniques that take advantage of the capability of Tat to bind heparin (known in the prior art and widely discussed in the specification (see Example 1), lyophilized and stored at -70 to -80°C and reconstituted before use in a buffer suitable for preventing oxidation and aggregation. (For example, containing a reducing agent such as dithiothreitol (DTT) and degassed, i.e., conditioned with a inert gas, such as nitrogen, in order to displace oxygen and to avoid oxidation in the presence of carrier proteins, such as serum albumin). (See legends to Tables 8, 16, 24, 32, and page 94, lines 12-13 of the specification.) These aspects of the process have been discussed in the prior art by the inventor herein (Ensoli et al J. Virol, 1993 and Nature, 1994; and in particular in ref. 26 of the present application, corresponding to Chang et al AIDS 1997.)

To prevent the attachment of the protein to surfaces, plastic tips and vials are rinsed in a buffer containing 0.1% bovine serum albumin or in medium supplemented with 15% fetal bovine serum. In addition, because Tat is also photo- and thermo-sensitive (Ensoli et al J. Virol, 1993; Chang et al AIDS, 1997), the handling of the protein is always performed in the dark and on ice. In summary, exposure to light and air determines the oxidation of Tat and loss of biological activity due to conformational changes, including multimerization and aggregation of the protein with loss of the substantially monomeric active form as shown by the inventor in the prior art (Ensoli et al J. Virol 1993; Chang et al AIDS 1997; Barillari et al Blood 1999 and J. Immunol. 1999) and below.

In fact, physico-chemical characterization of biologically active Tat, and Tat preparations deliberately exposed to light and air, clearly demonstrate that in the latter the substantially monomeric form of Tat is lost (see FIG. 1 attached). Of importance, *in vitro*

experiments with monocyte-derived dendritic cells (MDDCs, which are the most potent antigen presenting cells required to initiate the immune responses against antigens) from human donors demonstrate that only Tat in its substantially monomeric form is taken up by MDDCs (see FIG. 2 attached) and is capable of inducing them to express activation markers (see Table 1 attached) and to produce relevant cytokines and chemokines to drive Th1 responses (see FIG. 3 attached). Finally, only Tat in its substantially monomeric form is capable of activating MDDCs antigen presenting functions as assessed by measuring proliferative responses to allogeneic lymphocytes (see FIG. 4 attached) and to recall antigens, such as tetanus toxoid (see FIG. 5 attached). Notably, both in monkeys vaccinated with the Tat protein or infected with the SHIV89.6P, and in HIV-1-infected individuals, autologous MDDCs treated with substantially monomeric biologically active Tat are a strong stimulus in driving *in vitro* expansion of Tat-specific γIFN producing T cells, a marker of CTL activity, as assessed by ELISpot (see Table 2 attached). Thus, only substantially monomeric Tat is selectively and efficiently taken up by MDDCs and promotes MDDCs maturation and activation, enhancing their antigen presenting functions and capability of driving Th1 and CTL responses against Tat and other antigens as well. Therefore, only substantially monomeric Tat possesses the full immunological properties, which are required to induce, when given *in vivo* as a vaccine, broad (i.e., humoral and cellular immune responses, including CTLs) and effective anti-Tat immune responses in monkeys, and predictably in humans, as inferred by the results obtained *in vitro* with human MDDCs. It is of great importance to note that the entry of biologically active Tat in MDDCs occurs at a protein concentration as low as picomolar, whereas the effects reported by others, including

immunosuppression and apoptosis, have been observed at much higher (micromolar) concentrations of a presumably oxidized/aggregated and biologically inactive protein.

In fact, Applicant has never observed a toxic effect *in vitro* or *in vivo* (mice, monkeys) with biologically active Tat produced in accordance with the present invention and used *in vitro* at concentrations ranging from pico- to micro-molar or after inoculation in animals at doses up to 100 μg. Extensive toxicology studies have, in fact, been performed with biologically active Tat in small animals and monkeys and a large part of those results are present in the specification herein. Thus, in summary Applicant maintains that biologically active Tat is a protein in substantially monomeric form that results from production, purification and storage processes, which are disclosed herein in the prior art (Chang et al., AIDS 1997) and which are strictly required to obtain the biologically active Tat with the characteristics and activities as defined by the claims herein.

In summary, Applicant has found that, at the time the invention was made, only by using specific production, purification and storage protocols, namely, those disclosed in ref. 26 (Chang et al., AIDS 1997) in the present patent application, could a biologically active and stable Tat protein be obtained (see page 25, lines 5-14). Only the Tat protein prepared, purified and stored in accordance with the disclosed and claimed procedures herein could prevent or avoid oxidation and aggregation and, thus, permit its use as the active principle for a preventive and/or therapeutic vaccine against HIV infections and related symptoms and syndromes and only such form of Tat can perform the activities recited in the claims. Therefore, the only way to unequivocally identify this new type of Tat, i.e., biologically active, when isolated, is to characterize it by its physical form (i.e., substantially monomeric

versus aggregated) and functional activity, as recited in the claims. In fact, a Tat prepared according to standard and known techniques, when isolated, is unstable and easily inactivated and, therefore, is ineffective and is not commensurate with the scope of the present invention as recited in the claims herein. Elements or features 1) and 2) recited in independent claims 62, 73, and 76 as well as the claims dependent thereon, provided the only available tools to properly and unequivocally verify the biological activity of Tat at the time the subject application was filed. The mutants and fragments of Tat which show such features are to be considered as being within the scope of the present invention since one of ordinary skill in the art would be able to understand that "mutant" and "fragment" is intended in light of the teaching of the present invention and, in general, in the light of the meaning of those terms in the biological sciences.

The biologically active Tat made in accordance with the claimed invention has been found suitable by Applicant for use as a vaccine. In fact, in direct contrast with the commonly prevailing knowledge at the time the invention was filed, as well as the belief still existing today, according to which a biologically active Tat is harmful to animals and humans when injected, Applicant has found and demonstrated that a Tat protein in such form (i.e. not oxidized and not aggregated and, therefore, substantially monomeric and biologically active), provides results which are not harmful in small animals (mice and guinea pigs, see tables 3, 4 and 5 attached) and in monkeys (see safety data of the present application in example 4, pages 42-70, and particularly at page 43, lines 15-20 and page 51, lines 26-29). In addition, it induced a very strong antibody response (figs. 2A to 8 and 11-13 and tables 4, 9, 10, 19), a cellular immune response (figs. 9 and 10, and tables 5, 6, 8, 11, 12, 15, 16, 20, 21, 24,

27, 28, 31 and 32), and natural immune responses (tables 7, 14, 23 and 30), that were able to control experimental infection in monkeys with a highly pathogenic SHIV virus (as reported at Example 4). These data have proven so impressive and revolutionary with regard to the rationale of HIV vaccine development as to deserve and warrant publication in the highly respected peer-reviewed journal *Nature Medicine* (Cafaro et al., Nature Medicine, 1999). As a consequence of the above and in stark contrast with the "*contemporary knowledge in the art*", that "*would not allow one skilled in the art to use the claimed invention with a reasonable expectation of success*" and that still considers that "*biologically active Tat has known pathological activities*" and therefore cannot allow the skilled "*artisan to be confident of practicing the instant invention*", the Italian Government has sponsored and funded phase I clinical trials for both the preventive and the therapeutic approaches with the biologically active Tat protein as a vaccine against HIV/AIDS (see document of Ministry of Health and the Istituto Superiore di Sanità, attached hereto). Several international organizations are collaborating with Applicant to perform studies preliminary to trials with the biologically active Tat in their own populations. Thus, results obtained and presented herein, as well as results obtained after filing this application, have convinced many if not most scientists expert in AIDS research that vaccination with biologically active Tat is safe, immunogenic and able to control virus replication and disease onset, as disclosed and claimed in the present application.

It must be stressed that at the time of filing the present patent application, the entire scientific community was of one mind, namely that Tat produced by the processes described above was harmful. Nonetheless, Applicant, courageously and proceeding against the

commonly and generally accepted wisdom and perceived knowledge in the field, proceeded to test the biologically active Tat, as claimed herein, in monkeys (tests for immunogenicity and efficacy on inferior animals, such as mice, are not significant because immunogenicity in mice and humans differs qualitatively and because protection from infection can be evaluated only in nonhuman primates) and found it, most surprisingly and unexpectedly, to be non-toxic, immunogenic and efficacious in protecting monkeys from SHIV89.6P challenge. Applicant's findings under these circumstances, it is submitted, are the epitome of non-obviousness.

Retrospective epidemiological evidence indicates that a Tat-specific immune response (namely, anti-Tat antibodies and CTLs) in humans correlates with nonprogression to AIDS. In nonhuman primates protection from overt infection or disease progression has been recently obtained with a few vaccine candidates and it has been associated with the presence of both humoral and cellular immune responses, including CTLs. Evidence of the protective role played by the immune response stems also from the demonstration that in monkeys, depletion of any of the components of the immune response substantially modified the course of the retroviral infection (reviewed in: Ensoli & Cafaro, AIDS Clin. Rev. 2000/2001, 2000; Kent et al., Immunol. Rev. 2001). Therefore, based on these data and on historical evidence that immune response to microorganisms confers protection, it is conceivable for one skilled in the art to understand why the immune response to Tat, a gene product expressed very early after infection and critical for virus replication, may confer protection from overt infection and disease progression. That this is the case is also indicated by review articles

written by experts in the field after the filing of the present patent application (Mooij & Heeney, Vaccine 2001; Rappuoli, Vaccine 2001)

According to another aspect of the present invention, it has been found that, in principle, that many Tat mutants can be used to make a vaccine against AIDS and related syndromes. In fact, it is well-known to experts in the field that AIDS is the result of an infection produced by the HIV virus and that the HIV virus mutates rapidly, generating mutants which can still cause AIDS. Consequently, all these mutants produce the corresponding Tat mutants. However, because of the conservation of the amino acid sequence in the Tat functional domains, each of such Tat mutants is able to elicit an immune response against any variant of HIV.

Many published papers have demonstrated that Tat mutants are active if mutations spare the functional domains of the protein. In addition, even though mutations are present in the functional domains, Tat can still be active if other mutations are present in the LTR motif (that contains the target sequence of Tat), which can restore the biological activity of Tat. Therefore, one of ordinary skill in the art can easily envisage which mutants of Tat are still active and how to obtain them by standard site-directed mutagenesis techniques.

Claims 1-6, 8 and 57-59 stand rejected under 35 USC §101. This rejection is traversed.

To overcome the Examiner's rejection, the newly added claims are presented, all of which are supported in the as-filed specification of the now-cancelled claims. In particular, new independent claim 62 is presented as a "pharmaceutical composition" claim.

Insofar as the Examiner's assertions are concerned, to wit, *"the specification fails to teach, nor does it describe such use (biologically active Tat as a vaccine)", "to provide proof ... either clinical or in vitro or in vivo data ... can be used"*, Applicant is puzzled in that the specification does contain real data of *in vivo* tests which show the activity of a vaccine against HIV based on the biologically active Tat protein as disclosed and claimed, and the invention as claimed is based on such data and results.

The examples provide clear evidence that monkeys have been vaccinated against HIV virus. In Example 4, at pages 42-70 of the specification, nine monkeys were inoculated with the biologically active Tat protein. In this example two experiments were reported. In the first pilot experiment, three (3) monkeys were inoculated with the biologically active Tat protein in order to assess safety and immunogenicity. Clinical and immunological data confirmed that injection with biologically active Tat protein is safe, since no local (at the site of infection) nor systemic (alterations of hemato/chemical and immunological parameters) side effects were evidenced at the time of each injection, and also immunogenic, since both humoral and cellular response to Tat was elicited.

In the second experiment (from page 50, line 20 of the specification), six (6) monkeys were injected with the biologically active Tat. Results of this larger set of primates confirmed the absence of toxicity and the ability of Tat to elicit Tat-specific immune responses, both humoral and cellular, including generation of Tat-specific CTLs.

In addition, a viral challenge using the highly pathogenic SHIV89.6P virus was performed. Four (4) out of the six (6) monkeys were able to control virus replication, as measured by plasma viremia, cytoviremia, antigenemia and the presence of proviral DNA,

and normal CD4 T cell counts in comparison to the control monkeys and the two (2) vaccinated and "unprotected" monkeys. The same monkeys were still alive and in good health two (2) years after the challenge when the experimental protocol was terminated and they were sacrificed. Finally, new and more recent vaccine protocols in monkeys with the biologically active Tat have confirmed these data, which clearly demonstrate to one of ordinary skill in the art that the vaccine based on the biologically active Tat protein is safe, immunogenic and effective in controlling viral replication and disease progression also in humans. The data reported in the present patent application has, in fact, been, subsequently published in a very prestigious peer-reviewed journal, namely, *Nature Medicine*, (Cafaro et al., Nat. Med. 5:643-650, 1999), thus confirming that the results obtained were extremely convincing.

Furthermore, based on these data, phase I clinical trials with the biologically active Tat protein in both seronegative and seropositive individuals in Italy have been sponsored and funded by the Italian Government (see attached statement of the Italian Ministry of Health and the translated relevant sections) and will start in 2002. Thus, in conclusion, Applicant considers that in view of the large body of data, both those already disclosed in the present patent application and those presented herein, a reasonable expectation of success has been established sufficient to demonstrate the utility of the invention. Accordingly, since the rejection is deemed to have been overcome it should be withdrawn since a rejection under 35 U.S.C. §101 cannot be sustained.

The claims stand rejected under 35 USC § 112, second paragraph. The rejection is traversed.

Applicant maintains that the term "biologically active" refers to Tat protein, and corresponding mutants and fragments thereof that are capable of performing the limitations 1) and 2) recited in independent claims 62 and 73, as well as in the claims dependent thereon.

The sentence "*endothelial cells protein*" was a typographical error. It was intended to read "*endothelial cells*". Claims 62, 73, and 76 now recite "endothelial cells".

The new claims have been reworded in order to make it absolutely clear that they refer to a composition and to a therapeutic treatment. Since the §112, second paragraph, rejection has been overcome, its withdrawal is solicited.

The claims stand rejected under §112, first paragraph. This rejection is traversed.

Insofar as the rejection is based on nonenablement is concerned, when one of ordinary skill in the art reads about an immune response, he understands that the substance (Tat protein) that induces such response is a candidate for a vaccine composition. In fact, unless the Examiner has access to evidence still unknown to the scientific community, protection conferred by available vaccines is generally attributed to, and in some cases demonstrated by, the induction of specific immune responses to pathogenic microorganisms or their products. Moreover, a specific preparation and handling of the protein is disclosed, and it is also disclosed that it can be administered after being re-suspended in a biological fluid.

In addition, Applicant has provided evidence that administration of such preparation containing the HIV-1 Tat protein according to the schedule reported (see page 42, lines 17-23 and lines 27-28, page 50, lines 23-25, page 51, lines 1-2 and lines 5-11 of the specification) to nonhuman primates confers protection against a challenge with a pathogenic SHIV, a chimera between SIV and HIV. To date, monkeys are considered the only model to evaluate

the efficacy of an HIV vaccine, and demonstration of efficacy of a candidate vaccine in the monkey model constitutes a very strong rationale to proceed to testing in humans. Further, since vaccination was performed with the HIV-1 Tat, and a SHIV expressing the HIV-1 Tat was used for the challenge, Applicant has provided evidence of protection with a product suitable for use in humans as such, unlike many other HIV candidate vaccines that, in fact, are SIV vaccines and for which efficacy in humans can only be indirectly inferred.

Finally, the HIV-1 Tat used for monkeys' immunization has the same physical/chemical and biological characteristics as the Tat currently being produced in GMP (Good Manufacturing Practices) facilities for experimentation in humans and, therefore, the results from the reported monkey studies fulfil the criteria of the pre-clinical testing needed for filing a request for approval for clinical trials.

Still further, one of ordinary skill in the art could and would have readily identified those Tat variants and fragments with biological activity, as far as they correspond to the definition recited in the claims herein and as further clarified by the present reply. In addition, synthesis of these mutants is a relatively easy procedure for one of ordinary skill in the art. In fact, they can be either synthesized or produced by recombinant DNA methods or by site-directed mutagenesis (see "Molecular Cloning" Second edition and following, Sambrook J., Fritsch E.F., Maniatis T. Eds.)

Therefore, in the specification there exists a solid foundation which teaches both *how to make and how to use* the invention. In fact in the specification a reproducible method for producing biologically active Tat is given and reproducible tests showing the efficacy of Tat

as a vaccine are given. A person of ordinary skill in the art is thus provided with all the elements to successfully perform the invention.

As a consequence, the following Examiner's assertions are untenable *"the state of the art is such that the artisan would not be confident of practising the invention", "Applicant has not provided any convincing evidence ... without undue experimentation"*. Since the §112, first paragraph, rejection has been overcome, it should be withdrawn.

The claims stand rejected under §102(a) as anticipated by Frankel et al., U.S. 5,652,122. Applicant respectfully traverses this rejection.

Frankel teaches the use of Tat as a delivery system for molecules of interest in therapy. Frankel refers to "naturally-occurring amino acid sequence which is the same as that of naturally-occurring tat protein, its functional equivalent or functionally equivalent fragments thereof (peptides). Such functional equivalents or functionally equivalent fragments possess uptake activity into the cell and into the cell nucleus that is substantially similar to that of naturally-occurring tat protein" (col. 9, lines 5-11).

15 This is qualitatively (cell types taking up Tat: epithelial, monocytic and lymphocytic established tumor cell lines for Frankel, as compared to Applicant's primary endothelial, dendritic and KS cells, which are truly representative of Applicant's cells *in vivo*) and quantitatively (range of Tat concentrations at which uptake occurs: 2-50 μg/ml for Frankel versus 0.01-100 ng/ml for the Applicant) totally different and readily distinguishable from the definition of biologically active Tat as claimed by Applicant. Therefore, Frankel is ineffective as an anticipatory reference with respect to the claims of the present invention.

Furthermore, Frankel also states (col. 9, lines 65-67) that it is preferred to remove the Tat cystein-rich region in order to avoid problems. Proceeding in accordance with Frankel's process would eliminate the biological activity of Tat as claimed by Applicant. In addition, according to Frankel, "It is known that the second exon is not required for activity" (col 20, line 19-23). This, too, is different and distinguishable from the definition of biologically active Tat claimed by Applicant because the second exon of Tat and, in particular, the RGD motif therein is required for the efficient Tat uptake by dendritic cells and activated endothelial cells. Therefore, Frankel's teaching is exactly the opposite of, and completely contrary to, the claimed invention.

It is of the utmost importance to note that none of the Examples reported by Frankel utilizes a two exon Tat. Therefore, the Tat protein utilized by Frankel and the two exon Tat protein utilized by Applicant are utterly different and Frankel's disclosure cannot possibly be said to be anticipatory. Moreover, the preparation of the Tat disclosed in Frankel is traditional and would lead to an aggregated and oxidized Tat and, therefore, an inactive Tat, as indicated by the very high concentrations of the protein which are needed in order to observe any degree or level of uptake.

Moreover, Frankel did not provide any data relative to the physical/chemical characteristics of Tat, nor to the substantially monomeric form that is required to fulfil the definition of biologically active Tat as indicated herein. By contrast, the very high concentrations used by Frankel indicate that the Tat was neither substantially monomeric nor biologically active as claimed in this invention. Thus, the Tat produced by Frankel is very different from the Tat claimed herein and, therefore, cannot be said to anticipate the claims

of the present invention. Therefore, the rejection has been overcome and should be withdrawn since a *prima facie* case of anticipation under §102(a) has not been established.

The claims stand rejected under §103(a) for obviousness over Aldovinni. The rejection is respectfully traversed.

Aldovinni teaches obtaining a vector expressing Tat-3. The protein is then used in tests predictive of HIV infections (see page 3, lines 24-27, page 6, lines 16-18). Nothing is said explicitly nor can anything be inferred about the use of Tat as a vaccine. Moreover, the Tat, after its expression in Example 1, is grossly purified by resolution on a preparative polyacrylamide gel and then injected in rabbits in Example 2, page 11, lines 1-4. Nothing is said about taking care to protect the Tat against oxidation. Therefore, Aldovinni:

- teaches a method to prepare, purify, and to store a Tat, which is very different and distinguishable from the Tat according to the claimed invention;
- uses Tat in an animal model (rabbit) which is far removed from monkeys and man and not predictive of efficacy as a vaccine; and
- discloses a use in diagnosis and not in vaccination.

Consequently, the claimed invention distinguishes over the disclosure of Aldovinni and, accordingly the §103(a) rejection has been overcome and should be withdrawn since the Examiner has failed to establish a *prima facie* case of obviousness.

Since the rejections of record have been overcome, the issuance of a Notice of Allowance is respectfully solicited.

Please charge any fees which may be due herein to our deposit account No. 01-0035.

Respectfully submitted,

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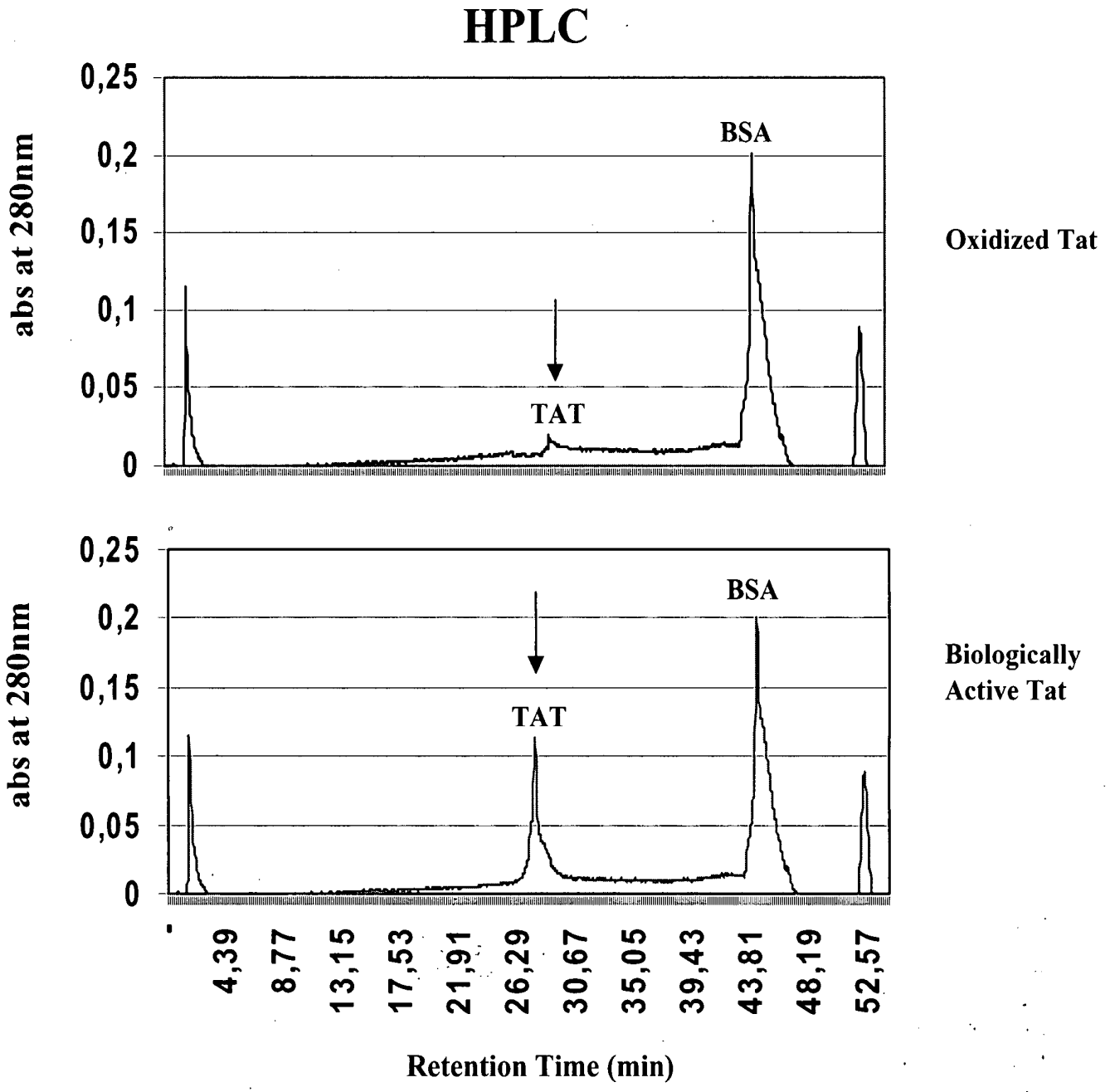
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0,05
0
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BSA
TAT
Oxidized Tat
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0,15
0,1
0,05
0
abs at 280nm
BSA
TAT
Biologically Active Tat
4,39
8,77
13,15
17,53
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30,67
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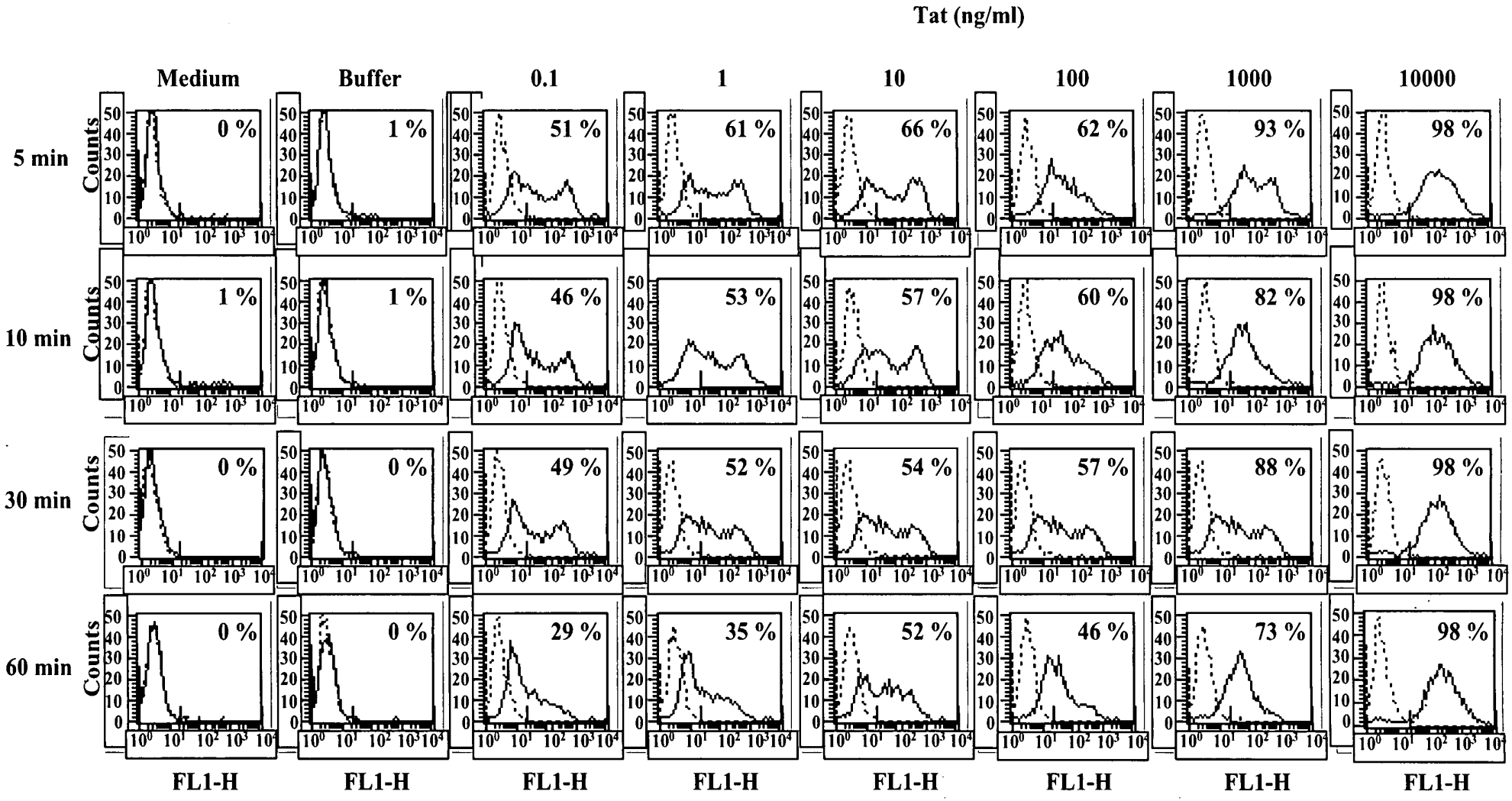
48,19
52,57
Retention Time (min)

Fig. 1

A


Tat (ng/ml)
Medium
Buffer
0.1
1
10
100
1000
10000
5 min
10 min
30 min
60 min
Counts
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0 %
1 %
51 %
61 %
66 %
62 %
93 %
98 %
1 %
1 %
46 %
53 %
57 %
60 %
82 %
98 %
0 %
0 %
49 %
52 %
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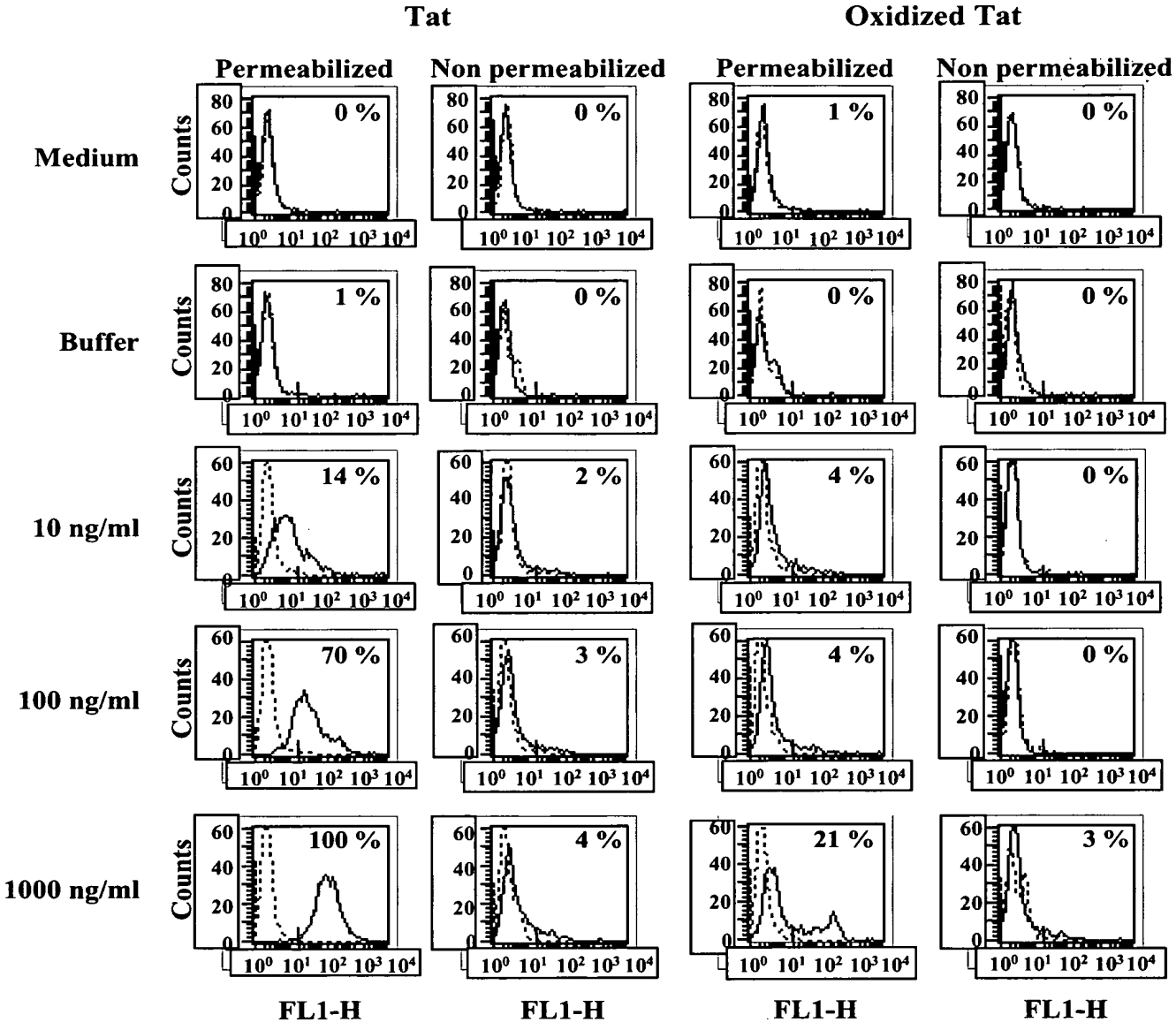
98 %

Fig. 2A

B


Tat
Oxidized Tat
Permeabilized
Non permeabilized
Permeabilized
Non permeabilized
Medium
Buffer
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100 ng/ml
1000 ng/ml
Counts
0 %
0 %
1 %
0 %
1 %
0 %
0 %
0 %
14 %
2 %
4 %
0 %
70 %
3 %
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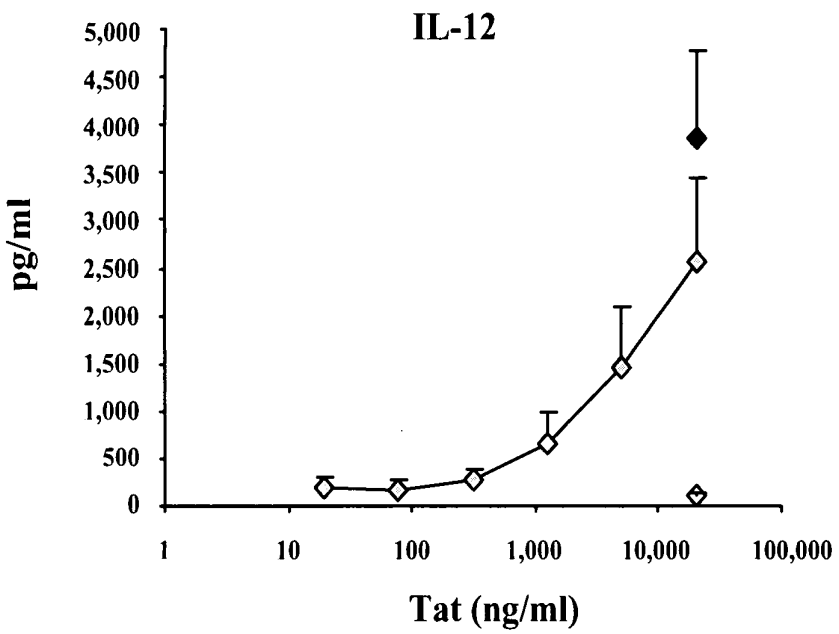
3 %
FL1-H
FL1-H
FL1-H
FL1-H

Fig. 2B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pg/ml
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4,500
4,000
3,500
3,000
2,500
2,000
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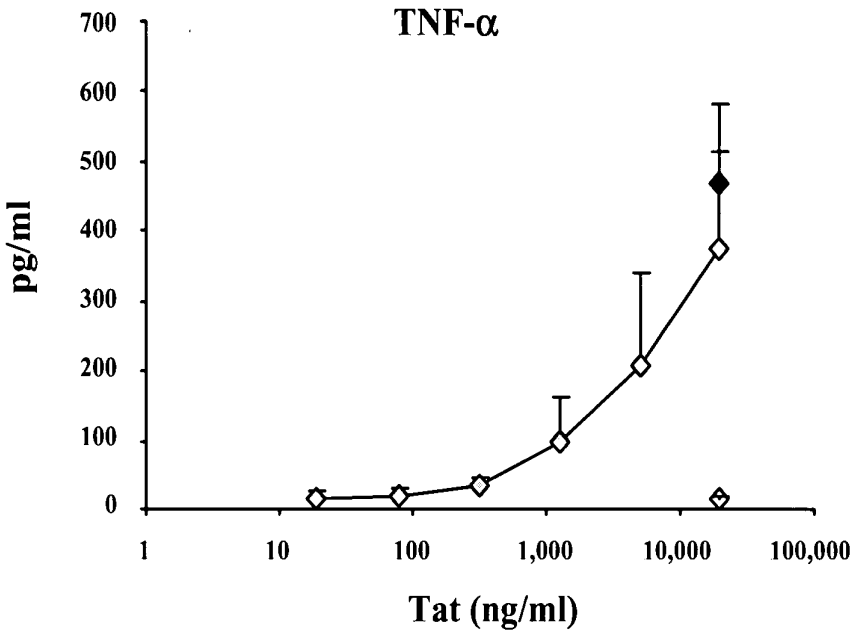
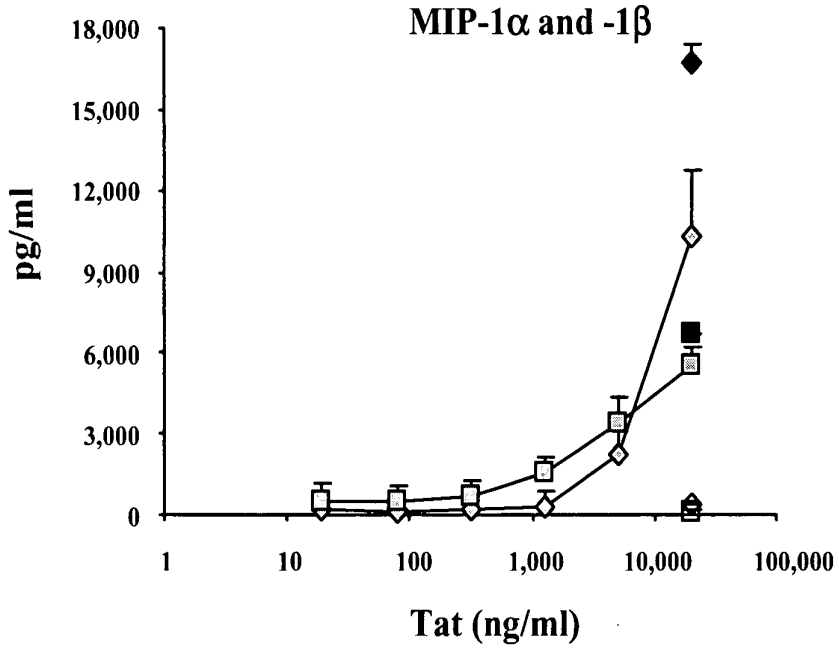
1,000
500
0
1
10
100
1,000
10,000
100,000
Tat (ng/ml)


TNF-α
pg/ml
700
600
500
400
300
200
100
0
1
10
100
1,000
10,000
100,000
Tat (ng/ml)


MIP-1α and -1β
pg/ml
18,000
15,000
12,000
9,000
6,000
3,000
0
1
10
100
1,000
10,000
100,000
Tat (ng/ml)

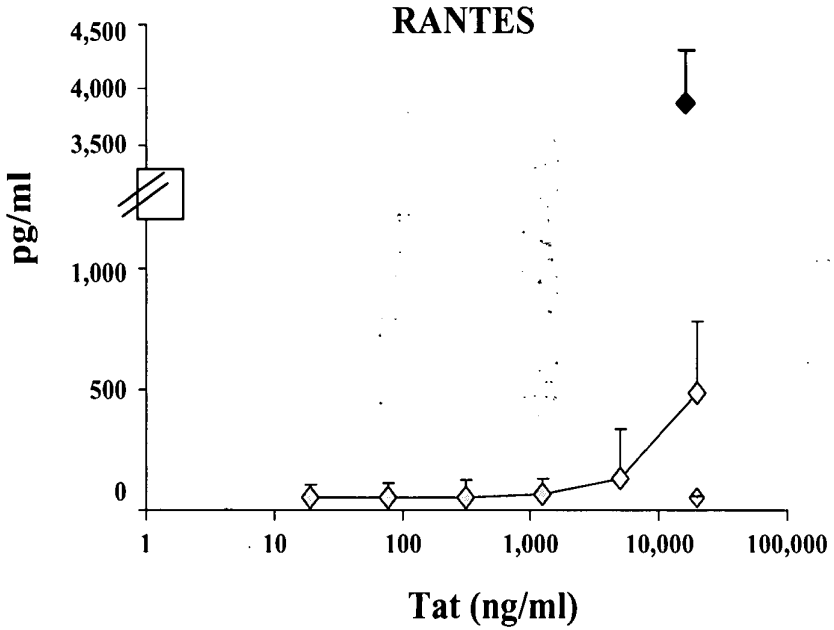

RANTES
pg/ml
4,500
4,000
3,500
1,000
500
0
1
10
100
1,000
10,000
100,000
Tat (ng/ml)

Fig. 3

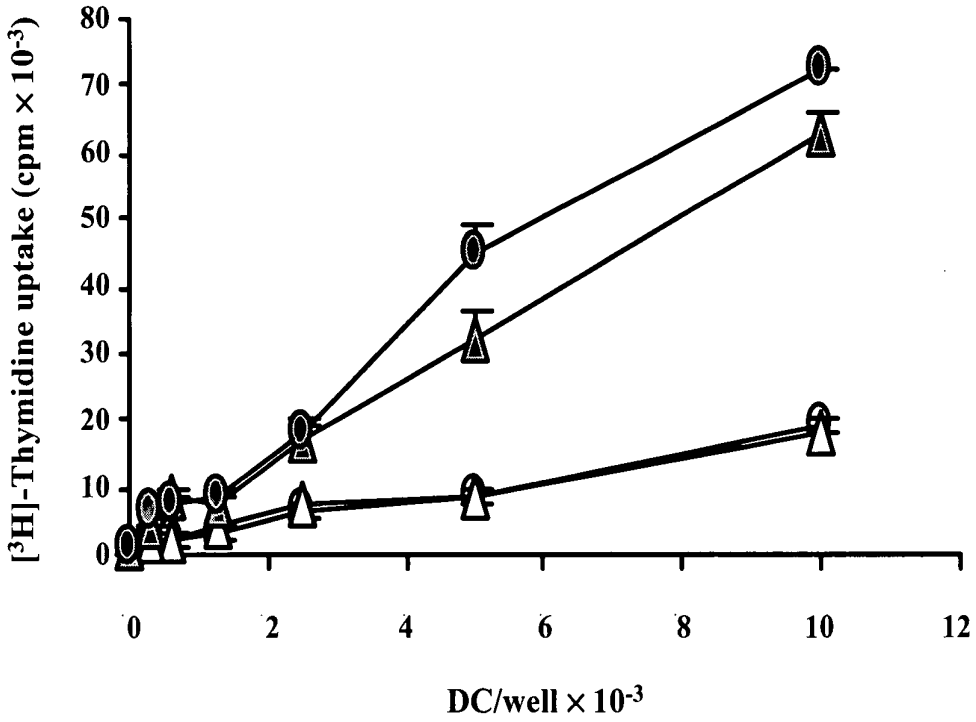

[3H]-Thymidine uptake (cpm × 10-3)
80
70
60
50
40
30
20
10
0
0
2
4
6
8
10
12
DC/well × 10-3

Fig. 4

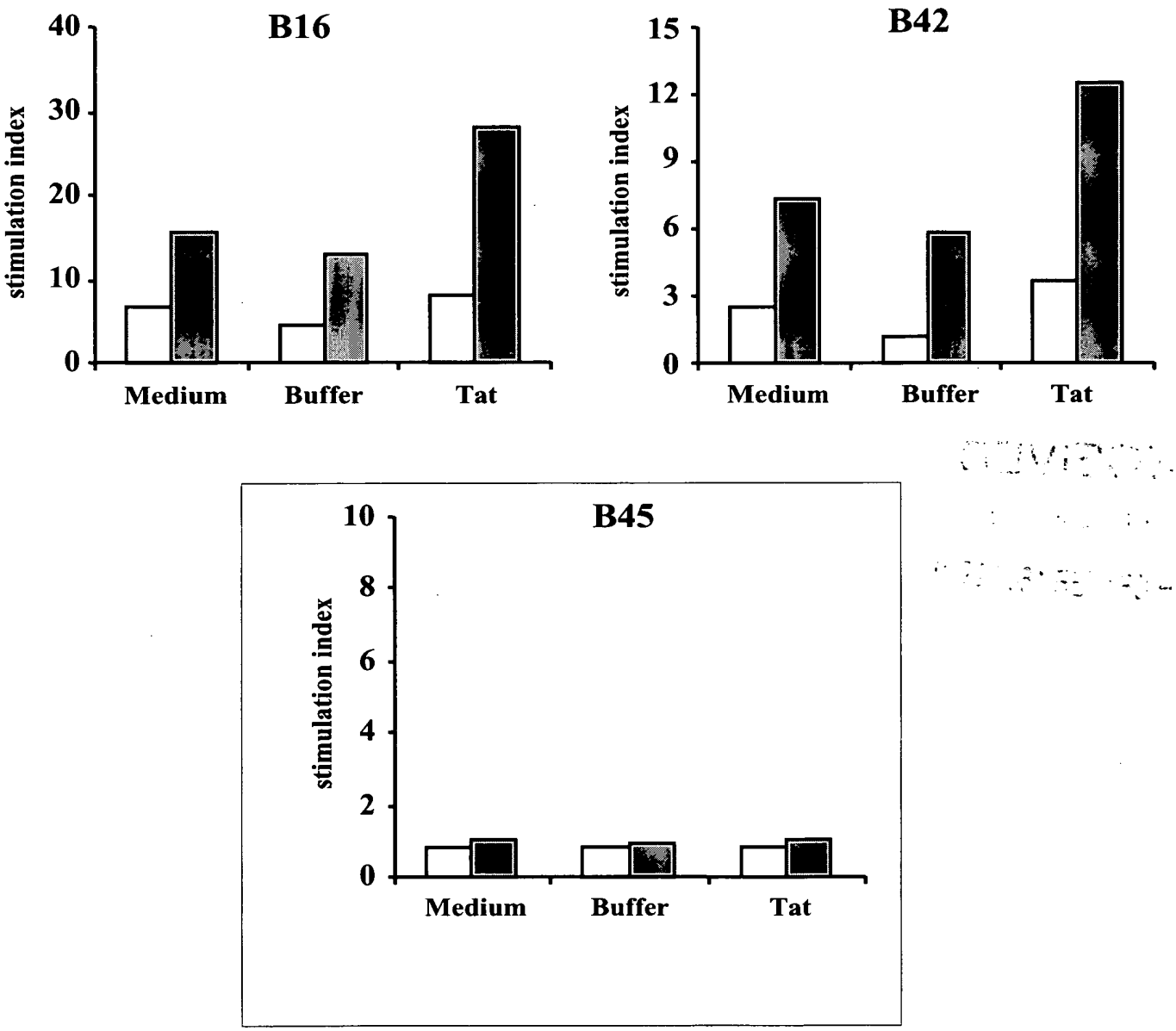

B16
stimulation index
40
30
20
10
0
Medium
Buffer
Tat
B42
stimulation index
15
12
9
6
3
0
Medium
Buffer
Tat
B45
stimulation index
10
8
6
4
2
0
Medium
Buffer
Tat

Fig. 5

Figure Legends

FIGURE 1. Comparison of biologically active Tat versus oxidized Tat by reversed phase HPLC analyses. Reversed phase HPLC analyses were performed using a Beckman Gold chromatographic system (Beckman, CA) equipped with a diode array detection system (Mod. 168). Twenty µg of purified Tat protein were injected onto a 5 µm Supelcosil LC 318 column (5cm x 4.6mm i.d.) protected by a Supelcosil LC 318 guard column (2cm x 4.6mm i.d., Supelco, PA). Solvent A was 0.1% (v/v) trifluoroacetic acid (TFA, Fluka, Switzerland) while solvent B was 0.1% (v/v) TFA in acetonitrile (Carlo Erba, Italy).

The gradient of solvent B used for protein separation was: 10% in 5 min, 10-40% in 30 min, 40-80% in 2 min, 80% in 5 min, 80-10% inl min and 10% in 2 min. The flow rate was 1 ml min^{-1} and the protein detection was performed at 214nm and at 280 nm.

FIGURE 2A and 2B. Native Tat is efficiently and selectively taken up by MDDC but it is lost by oxidation/inactivation of the protein. **A**, MDDC were incubated with serial concentrations (0.1 to 10,000 ng/ml) of the native Tat protein, medium or reconstitution buffer for 5, 10, 30 and 60 min, processed, washed, fixed and permeabilized as described in materials and methods. Intracytoplasmatic Tat content was evaluated by flow cytometry after staining with specific affinity-purified rabbit anti-Tat polyclonal Ab (or isotype control) followed by secondary FITC-conjugated anti-rabbit Ab. The percentage of positive cells (as compared to isotype stained samples) is reported into the boxes. ,whose levels of Tat uptake were min minpositive cells . Results are from a representative experiment and have been reproduced with at least 10 donors.

B, MDDC were incubated for 10 min in the presence of the native or the oxidized (by exposition to light and air for 18 h) Tat protein (10 to 1000 ng/ml) and processed as reported above. The percentage of positive cells and the mean fluorescence intensity (MFI) are reported into the boxes.

FIGURE 3. Tat enhances the production of the cytokines IL-12, TNF-α and the β-chemokines MIP-1α, MIP-1β and RANTES by MDDC. Supernatants of cells exposed to serial concentrations of Tat (19 to 20,000 ng/ml), reconstitution buffer, complete medium or LPS (positive control) for 18 hrs, were assayed for the levels of IL-12 (diamonds)- TNF-α(diamonds), MIP-1α (diamonds), MIP-1β (squares) and RANTES (diamonds). Empty and dark markers represent values from cells treated with buffer and LPS respectively. Data are reported as the mean values (± SEM) from eight different donors and are expressed in pg/ml. A very poor or no cytokine or β-chemokine production was induced by oxydized Tat (data not shown).

FIGURE 4. Tat enhances allogeneic Ag presentation by MDDC. MDDC were exposed for 18 h to complete medium (containing 5% FBS) (empty circles), LPS (closed circles), reconstitution buffer (empty triangles) or Tat (10 μg/ml) (closed triangles) and cultured together with allogeneic PBL. ^{3}[H]-thymidine uptake was measured after 6 days of culture to evaluate lymphocyte proliferation. Data are from a representative experiment and have been reproduced with other three different donors. Mean and SEM are reported.

FIGURE 5. Tat increases TT-specific presentation by MDDC to primed PBL enhancing specific T cell responses. MDDC from 3 healthy donors [(B16, B42 and B45) two of them (B16, B42) responsive to TT in proliferation assays] were exposed for 18 h to Tat (10 μg/ml), reconstitution buffer or culture complete medium (containing 5% FBS) and cultured together with autologous lymphocytes (ratio 1:20) in the absence (empty columns) or in the presence (grey columns) of TT (5 μg/ml). ^{3}[H]-thymidine uptake was measured after 6 days of culture to evaluate lymphocyte proliferation. Values are expressed as stimulation index (S.I.).

Table I. Native (A) but not oxidized-inactivated (B) Tat enhances the expression of HLA and costimulatory molecules on MDDC

A.

	Donor Code	Mean fluorescence intensity		% increase vs control	
		Medium	Buffer	Tat	LPS
HLA-ABC	B 19	392	332	78.3	74.0
	B 24	291	309	49.2	140.9
	B 26	304	297	15.8	32.6
	B 38	130	127	63.8	168.5
	B 53	276	272	2.2	48.9
	B 55	251	259	13.9	9.2
HLA-DR	B 19	145	192	27.1	84.8
	B 24	164	143	60.8	48.8
	B 26	175	177	60.5	60.6
	B 38	464	366	98.1	85.6
	B 40	1040	1127	38.9	58.9
	B 43	415	408	27.7	ND
	B 44	687	692	31.4	ND
	B 45	847	797	40.4	ND
	B 53	471	493	94.7	94.5
	B 55	313	303	47.2	30.7
CD40	B 19	53	51	23.5	77.4
	B 24	60	58	41.4	103.3
	B 26	42	43	16.3	45.2
	B 38	70	62	95.2	64.3
	B 40	85	76	60.5	76.5
	B 43	48	48	-6.3	ND
	B 44	69	68	14.7	ND
	B 45	87	76	18.4	ND
	B 53	82	80	52.5	75.6
	B 55	34	36	38.9	14.7
CD80	B 19	8	8	62.5	350.0
	B 24	16	16	43.8	143.8
	B 26	10	9	44.4	90.0
	B 38	21	19	100.0	109.5
	B 40	27	23	73.9	63.0
	B 43	9	8	25.0	ND
	B 44	18	18	27.8	ND
	B 45	21	21	52.4	ND

CD83	B 19	7	6	166.7	442.9
	B 24	9	7	257.1	200.0
	B 26	8	9	55.6	62.5
	B 38	5	6	233.3	520.0
	B 40	12	10	310.0	233.3
	B 43	8	9	22.2	ND
	B 44	9	13	7.7	ND
	B 45	7	10	90.0	ND
	B 53	3	6	150.0	433.3
	B 55	6	5	480.0	ND
CD86	B 19	81	76	82.9	133.3
	B 24	35	41	48.8	77.1
	B 26	128	129	48.1	55.5
	B 38	95	103	266.7	288.0
	B 40	25	24	95.1	144.0
	B 43	75	74	41.9	ND
	B 44	70	75	80.0	ND
	B 45	51	50	166.0	ND
	B 53	15	14	535.7	693.3
	B 55	37	40	97.5	32.4

B.

	Medium (MFI)	**Buffer (MFI)**	**Tat (% increase vs control)**	
HLA-DR	313	303	46.9	Native
			10.6	Oxidized
CD40	34	36	38.9	Native
			8.3	Oxidized
CD83	6	5	480.0	Native
			40.0	Oxidized
CD86	37	40	97.5	Native
			15.0	Oxidized

Cells were exposed for 18 h to native or oxidized-inactivated Tat (0.02 to 20 μg/ml), reconstitution buffer, complete medium, or LPS (10 μg/ml), stained with fluorochrome-conjugated mAb and then analyzed by flow cytometry. In panel A the expression of the surface molecules on MDDC from 10 different donors is reported as the percentage increase of the mean fluorescence intensity (MFI) of cells exposed to Tat (at the dose

which gave the maximum increase) or LPS as compared to the MFI of those exposed to Tat buffer or medium, respectively.

Data with oxidized versus native Tat on a representative donor are shown in panel B. MDDC cultured with LPS were used as the positive control for the induction of HLA and costimulatory molecules.

MDDC cultured in presence of native or oxidized Tat were always viable, not differing from those treated medium or reconstitution buffer.

Table 2. IFN-γ ELISpot. Number of spots (for 10^6 PBMCs) in response to the Tat protein versus buffer (control) in Tat-vaccinated or control monkeys challenged with SHIV89.6P or in a human infected with HIV at the time of the bleeding (day 0) or after 14-20 days of restimulation by co-colture with DC primed with the biologically active Tat protein.

Monkey No.	Status	Day 0	Day 14-20
		Tat/control	
1	Vaccinated	15/15	240/10
2	Vaccinated	10/15	420/10
3	Vaccinated	20/10	160/10
4	Vaccinated	15/5	40/20
5	Vaccinated	ND	180/20
6	Vaccinated	1/5	>1000/20
7	Vaccinated	5/5	100/20
8	Vaccinated	60/5	480/20
9	Vaccinated	45/10	200/20
10	Control	10/5	10/10
11	Control	15/10	30/10
12	Control	5/5	320/20
13	Control	5/5	20/20
14	Control	5/5	20/20
15	Control	5/5	20/20
16	Control	5/5	20/20
17	Control	5/5	40/20
18	Control	10/5	20/20
19	Control	5/5	20/20
20	Control	5/5	20/20
Human			
1	HIV-1 infected	69/10	1400/300

Table 3. Safety results with biologically active Tat protein upon inoculation in nude mice as compared to a lesion-inducing factor (bFGF).

N. Animals Tested	Inoculation	Route	Dosage	% Vascular lesions
136	Buffer	subcutaneous	-	0
59	bFGF	subcutaneous	0.1 μg	0
43	bFGF	subcutaneous	1 μg	44
15	bFGF	subcutaneous	10 μg	100
19	bFGF	subcutaneous	30 μg	100
10	bFGF	subcutaneous	90 μg	100
4	Tat protein	subcutaneous	0.1 μg	0
5	Tat protein	subcutaneous	1 μg	0
105	Tat protein	subcutaneous	10 μg	0
10	Tat protein	subcutaneous	30 μg	0
6	Tat protein	subcutaneous	60 μg	0
10	Tat protein	subcutaneous	100 μg	0

Table 4. Safety results with biologically active Tat protein upon inoculation in Guinea Pigs as compared to a vascular permeability factor (VEGF).

N. Animals Tested	Inoculation	Route	Dosage	Fold increase vs. control % Vascular permeability
4	Buffer	subcutaneous	-	0
4	VEGF	subcutaneous	0.1 μg	2
4	VEGF	subcutaneous	1 μg	4.3
9	Tat protein	subcutaneous	0.1 μg	0
10	Tat protein	subcutaneous	1μg	0
4	Tat protein	subcutaneous	10 μg	0

Table 5. Safety results of biologically active Tat upon immunization of different strains of immunocompetent mice by different routes of administration in the absence or presence of adjuvants

No. and type of mice strain tested	Immunization schedule (Days)	Route	Dosage	Local effects	Systemic effects
5 C57BL/6 mice	0 and 14	subcutaneous	10 μg of Tat	None	None
5 C57BL/6 mice	0 and 14	subcutaneous	10 μg of Tat + Alum	None	None
5 C57BL/6 mice	0 and 14	subcutaneous	10 μg of Tat + CFA	None	None
10 BALB/ c mice	0, 14 and 45	intraperitoneal	5 μg (at day 0) with complete FA 5 μg (at day 15 and 45) with incomplete FA	None	None
2 BALB/ c mice	0 and 10	intraperitoneal	10 μg (at day 1) with complete FA 5 μg (at day 10) with incomplete FA	None None	None None
5 BALB.B mice	0, 7, 14 and 21		10 μg of Tat	None	None
5 BALB.B mice	0, 7, 14 and 21	intranasal	10 μg of Tat + LT	None	None
5 BALB/c mice	0, 7, 14 and 21	intranasal	10 μg of Tat	None	None
5 BALB/c mice	0, 7, 14 and 21	intranasal	10 μg of Tat + LT	None	None
5 C57BL/6 mice	0, 7, 14 and 21	intranasal	10 μg of Tat	None	None
5 C57BL/6 mice	0, 7, 14 and 21	intranasal	10 μg of Tat + LT	None	None
5 C57BL/6 mice	0, 7, 14 and 21	intranasal	10 μg of Tat + CT	None	None